SENSITIZATION OF LYMPHOCYTES AGAINST POOLED ALLOGENEIC CELLS

I. Generation of Cytotoxicity Against Autologous Human Lymphoblastoid Cell Lines*

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"Abnormal" cells such as vaccinia virus infected cells (1) and many tumor cells (2, 3), appear to express additional (as compared with normal cells of that strain or individual) antigens that are cross-reactive with alloantigens detected serologically on normal cells of other strains or individuals. Tumor cells may also express antigens cross-reacive with target antigens of foreign haplotypes against which cell-mediated immune responses can be generated (2, 4–6).

In vitro sensitization of responding human lymphocytes with a pool of allogeneic normal cells from 20 individuals results in the generation of cytotoxic T lymphocytes that lyse target cells from individuals that differ from the responding individual with regards to determinants recognized by cytotoxic lymphocytes, i.e. CD antigens[1] (7). Included in such an allogeneic pool of lymphocytes may be essentially all the CD-like antigens of the species. Whether sensitization with the pool may be an effective means to generate cytotoxic cells directed against syngeneic or autologous abnormal target cells could be tested by using human lymphoblastoid cell lines (LCL) derived from lymphocytes transformed by Epstein-Barr virus (EBV) since LCL cells express target antigens recognized by lymphocytes sensitized against autologous LCL cells (8, 9).

The results reported herein demonstrate that sensitization of lymphocytes with the pool of allogeneic normal cells gives rise to effector cells cytotoxic to autologous LCL cells but not to autologous normal lymphocytes nor phytohemagglutinin (PHA) induced blasts. In contrast, sensitization of lymphocytes with cells from single allogeneic individuals rarely leads to the generation of effector cells cytotoxic for autologous LCL cells.

Materials and Methods

Lymphocytes were isolated from heparinized peripheral blood from normal healthy adults by Ficoll-Hypaque sedimentation. For some experiments, lymphocytes were fractionated into T-cell-enriched and B-cell-enriched populations by rosetting with sheep erythrocytes (SRBC), centrifuging on a Ficoll-Hypaque gradient, and isolating the cells at the interface (depleted of T cells) and

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[1] *Abbreviations used in this paper*: LCL, lymphoblastoid cell lines; EBV, Epstein-Barr virus; CD antigens, those antigens detected by cytotoxic lymphocytes.

TABLE I
*Lysis of Autologous and Allogeneic LCL Cells after Pool Sensitization**

Responder cells	Stimulating cells	Number of effector cells per target cell	Target cells					
			A-LCL	A	S-LCL	S	C-LCL	C
			% Specific ^{51}Cr release (±SD)					
A	$Pool^{20}_x$	35	46.7 ± 5.7	−2.8 ± 3.7	92.3 ± 7.6		87.4 ± 6.5	
A	$A\text{-}LCL_x$	60	41.1 ± 19.1					
S	$Pool^{20}_x$	35	82.4 ± 9.2		17.9 ± 3.3	2.8 ± 3.3	85.9 ± 7.2	64.5 ± 3.4
S	$S\text{-}LCL_x$	60			33.4 ± 2.4			
C	$Pool^{20}_x$	35	94.0 ± 11.8	70.1 ± 9.1	91.4 ± 7.6	44.3 ± 5.4	5.3 ± 3.2	−2.8 ± 1.6
C	$C\text{-}LCL_x$	60					70.2 ± 3.5	

* Lymphocytes from individuals A, S, and C were stimulated with X-irradiated autologous LCL cells (designated A-LCL, S-LCL, or C-LCL) or a pool of 20 allogeneic stimulating cells and lysis of the LCL cells and normal lymphocytes (A, S, and C) was measured on day 7. Lymphocytes from A, S, and C cultured in media alone caused −3.0–2.3% ^{51}Cr release from autologous LCL cells.

the pellet containing rosetted cells (more than 95% T cells). The micromethod used for generating and assaying allogeneically induced cytotoxic human lymphocyte responses in vitro has been recently described (10). In brief, 1×10^5 responding lymphocytes/well were cultured with 1×10^5 X-irradiated (2,500 rads) stimulating cells from single unrelated individuals, with "pool" stimulating cells prepared by pooling equal numbers of lymphocytes from 5–20 unrelated individuals (7, 11), or with 2×10^4 X-irradiated (4000 rads) autologous LCL cells. LCL cells were generated by infecting peripheral blood lymphocytes with EBV (12) and were grown in RPMI-1640 containing 25 mM Hepes buffer and 10% heat inactivated normal human serum; the LCL cells were used for experiments within 6 mo after their establishment and were found to be free of mycoplasma contamination. In some experiments, effector cells were generated in upright 50 ml tissue culture flasks as previously described (13) by using 8×10^6 responding lymphocytes and 8×10^6 X-irradiated pooled allogeneic stimulating cells or 1.5×10^6 X-irradiated autologous LCL cells. ^{51}Cr release assays were performed 7 days after the onset of mixed culture and were terminated after 7 h. The percent specific ^{51}Cr release was calculated as previously described (10). All sensitizations and cytotoxic assays were performed in medium consisting of RPMI-1640 containing 25 mM Hepes Buffer and 20% heat-inactivated normal human serum.

Results

Shown in Table I are results of an experiment in which lymphocytes of three different individuals were cultured with X-irradiated cells of a pool consisting of 20 members for 7 days and were tested for their ability to lyse allogeneic LCL cells, autologous LCL cells, and autologous cultured normal lymphocytes. Lymphocytes from individuals A and S after pool sensitization significantly lysed autologous LCL cells as well as allogeneic LCL cells. Pool sensitized lymphocytes, however, failed to kill autologous normal lymphocytes, consistent with the findings of Martinis and Bach (7). Although LCL cells from individual C stimulated autologous lymphocytes to differentiate into cytotoxic lymphocytes capable of lysing the autologous LCL cells (as did autologous sensitization with A and S), pool sensitization of lymphocytes from individual C did not lead to significant lysis of autologous LCL cells. Lymphocytes from individuals A, S, and C when cultured without stimulating cells failed to kill autologous LCL cells.

Whereas pool sensitization is an effective means for generating T lymphocytes cytotoxic for autologous LCL cells in three of the four individuals tested, cultured autologous unfractionated lymphocytes, lymphocytes enriched for T

TABLE II

*Ability of Pool Sensitized T Lymphocytes to Lyse Autologous LCL Cells and Their Failure to Lyse Autologous B Cells, T Cells, and PHA Blasts**

	Responding T lymphocytes	Stimulating cells	Target cells				
			H-LCL	H-T Cells	H-B Cells	Allogeneic LCL	
			% specific ^{51}Cr release (±SD)				
Exp. 1	H	$Pool^{20}_x$	20.8 ± 8.9	−1.8 ± 9.4	2.9 ± 9.7	35.9 ± 5.2	
	D	H_x		61.9 ± 6.9	65.2 ± 11.6		
Exp. 2			S-LCL	S-Cultured lymphocytes	S-PHA Blasts	M-Cultured lymphocytes	M-PHA Blasts
	S	$Pool^{20}_x$	15.3 ± 3.1	−2.7 ± 2.0	−1.3 ± 3.9	23.9 ± 5.0	13.3 ± 4.7
	M	$Pool^{20}_x$		43.0 ± 5.6	47.2 ± 4.8	−0.5 ± 3.7	−4.3 ± 1.7

* Responding T lymphocytes were stimulated with X-irradiated pool of 20 allogeneic cells or X-irradiated cells of individual H. CML assays were performed on day 7 by using 40 effector cells:target cells. T-enriched and B-enriched cells from individual H (designated H-T and H-B) were isolated and cultured for 7 days before use as target cells. PHA blasts were used 60 h after incubating lymphocytes with PHA.

cells or B cells, and autologous PHA blasts are not lysed by the pool sensitized cells (Table II). Results of Exp. 1 show that sensitization of T cells from individual H gave rise to CTLs capable of lysing allogeneic as well as autologous LCL cells but not autologous normal T-enriched nor normal B-enriched cells, that were, however, sensitive to lysis by T cells from individual D after stimulation with cells of individual H. Similar results were obtained in another experiment. Shown in Exp. 2 is the finding, with appropriate controls, that T cells of individuals after pool sensitization lysed autologous LCL cells but failed to lyse autologous normal lymphocytes or PHA blasts.

Shown in Table III are results of two experiments to determine whether sensitization of lymphocytes with cells from individual members of the pool would be equally efficacious in terms of generating cytotoxic lymphocytes directed against autologous LCL cells. In Exp. 1, lymphocytes from individual S were sensitized in separate mixed cultures with cells of 10 unrelated individuals, designated 1–10, with three different pools of allogeneic normal cells consisting of 5, 10, and 20 members, respectively, and with autologous LCL cells. No cytotoxicity for LCL cells was observed on day 5 or 6 (data not shown). Stimulation of S with cells of individual 2 resulted, by day 7, in the generation of cytotoxic cells that lysed autologous LCL cells, however, very low or insignificant amounts of lysis of the LCL cells occurred by lymphocytes sensitized to any of the other nine individuals. In contrast, sensitization of S with the pool of cells from 20 members resulted in the same level of cytotoxicity on autologous LCL cells as did sensitization with S's own LCL cells and resulted in a greater cytotoxic response than did sensitization with pools consisting of cells from 5 or 10 members.

In a second experiment, shown in Table III, sensitization of cells of individual S to a pool of either 8 or 18 members resulted in significant cytotoxicity for his autologous LCL cells as well as target cells from all 6 members of the pool tested. In contrast, effector cells generated in each of the eight individual mixed cultures did not lyse autologous LCL cells in any of the eight cases although

TABLE III

Lysis of Autologous LCL Cells and Allogeneic Cells after Allo- or Pool-Sensitization

Exp. 1			Exp. 2 Target cells						
Effector cells*	% specific ^{51}Cr release on S's LCL target cells‡	Effector cells	S's LCL cells	A	B	D	E	G	H
			% specific ^{51}Cr release ± SD‖						
$S(1)_x$	+4.7	$S(A)_x$	0.0 ± 2.7	33.5					
$S(2)_x$	+17.5	$S(B)_x$	1.9 ± 3.0		43.7				
$S(3)_x$	+3.9	$S(C)_x$	2.8 ± 3.9						
$S(4)_x$	−2.7	$S(D)_x$	1.9 ± 4.3			50.9			
$S(5)_x$	+9.1	$S(E)_x$	3.7 ± 4.4				92.9		
$S(6)_x$	+5.8	$S(F)_x$	0.1 ± 2.7						
$S(7)_x$	+5.3	$S(G)_x$	−0.8 ± 3.8					39.3	
$S(8)_x$	−0.7	$S(H)_x$	2.3 ± 5.0						37.1
$S(9)_x$	0	$S(Pool^8)_x$	18.7 ± 3.2			53.9			
$S(10)_x$	0	$S(Pool^{18})_x$	23.0 ± 2.6	18.5	30.3		101.6	45.9	112.9
$S(pool^5)_x$	+9.6	$S(S\text{-}LCL)_x$	24.6 ± 3.1						
$S(pool^{10})_x$	+16								
$S(pool^{20})_x$	+22.3								
$S(S\text{-}LCL)_x$	+20.5								

* Lymphocytes were sensitized with X-irradiated normal lymphocytes from individuals 1–10, with pooled allogeneic cells from 5, 10, or 20 members (designated $pool^5$, $pool^{10}$, $pool^{20}$) or autologous LCL cells in microwells for 7 days.

‡ Standard deviations of the percent ^{51}Cr release ranged from 1.4 to 5.2%.

§ Lymphocytes were sensitized with X-irradiated normal lymphocytes from individuals designated A–H, with pooled cells from 8 of 18 individuals, or with autologous LCL cells and cytotoxicity on the allogeneic target cells derived from members of the pool and autologous LCL cells were assayed on day 7 by using a ratio of 25 effector cells:target cell.

‖ SD ranged from 1.1 to 6.2% ^{51}Cr release.

these same effector cells were highly cytotoxic for target cells derived from the sensitizing cell donors.

Since sensitization of lymphocytes from S with cells from individual 2 (Table III) resulted in the generation of cytotoxic cells directed against autologous LCL cells, cold target inhibition studies were performed to determine whether lysis of LCL cells after pool sensitization was due solely to the presence of cells from individual 2 in the pool. When cells of S were sensitized to the pool and then tested for their ability to lyse ^{51}Cr-labeled autologous LCL cells, the addition of unlabeled LCL cells blocked lysis whereas unlabeled target cells from individual 2 did not (Fig. 1A). In contrast, when lymphocytes from S were sensitized with cells from individual 2, lysis of the autologous LCL cells was blocked equally well with unlabeled target cells from individual 2 or unlabeled LCL cells (Fig. 1B). Lysis of target cells from individual 2 by lymphocytes sensitized to the pool or to cells of 2 was not blocked by the LCL cells but was by unlabeled target cells from individual 2.

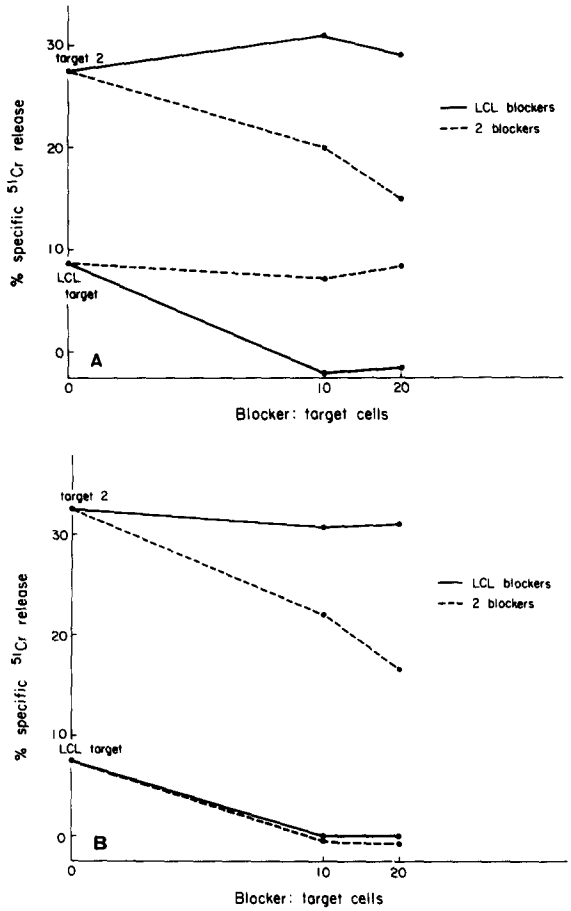

FIG. 1. Ability of unlabeled cells to block ^{51}Cr release from LCL cells of S on target cells from individual 2. Lymphocytes from individual S were sensitized to a pool of 20 members (A) or to cells from individual 2 (B) and were tested for their ability to lyse ^{51}Cr labeled LCL cells or target cells from individual 2 at a ratio of 30 effector cells: target cell in the presence of varying numbers of unlabeled ("blockers") LCL cells or cells from individual 2.

Discussion

The data presented in this paper demonstrate that in vitro sensitization of human lymphocytes with a pool of allogeneic normal human lymphocytes results in the development of effector cells that lyse autologous EBV transformed lymphoblastoid cells in three of four individuals tested. We have extended our previous findings (7) by demonstrating that T cells can respond to allogeneic pooled cells by differentiating into CTLs capable of lysing allogeneic normal lymphocytes, LCL cells, and PHA blasts but not autologous cultured lymphocytes enriched for T cells or B cells, nor autologous PHA blasts.

An occasional allogeneic normal cell appears to carry determinants cross-reactive with target antigens on autologous LCL cells although most allogeneic normal cells would not appear to do so. Sensitization with the cells of individual 2 (Table III and Fig. 1B) resulted in lysis of LCL cells autologous with the

responding cell donor. Results of blocking experiments demonstrated that lymphocytes sensitized to cells of individual 2 were not blocked from lysing 2's target cells by LCL cells (see Fig. 1B) and that after pool sensitization, lysis of the LCL cells was blocked only by LCL cells and not by cells of individual 2 (see Fig. 1A). We would interpret these findings to indicate that target antigens on the autologous LCL cells include determinants in addition to those shared by individual 2, and that the shared determinants represent a minority of all those that can be recognized by S's lymphocytes after pool sensitization.

It would seem that there are at least three mechanisms by which antigens expressed on LCL cells might cross-react with alloantigens. First, as a result of EBV infection or morphological transformation there may be derepression of genes coding for antigens that are expressed on normal cells of other members of the species (14, 15). Second, EBV may code for cell membrane determinants that partially cross-react with many alloantigens. Third, any genetic or phenotypic modification of CD antigens might result in expression of target antigens that would be cross-reactive with alloantigens (16). Use of the pool may have general applicability in terms of generating effector cells cytotoxic for autologous or syngeneic virus infected or morphologically transformed cells (16).

Summary

Lymphocytes sensitized in vitro to a pool of X-irradiated allogeneic normal lymphocytes from 20 individuals develop cytotoxic activity for autologous human lymphoblastoid cells (LCL). Whereas pool sensitized T lymphocytes lyse autologous LCL cells, they fail to lyse autologous B-enriched or T-enriched normal target cells nor autologous phytohemagglutinin (PHA) blasts. In contrast to pool sensitization, stimulation with normal cells of single allogeneic individuals rarely led to development of cytotoxicity against autologous LCL cells. We conclude that human Epstein-Barr virus transformed LCL cells express target antigens cross-reactive with allogeneic target antigens expressed on normal cells and that sensitization with a pool of allogeneic cells is an effective means of generating effector cells directed against autologous abnormal cells.

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